

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

DESCRIPTION

Luciferase and Methods for Measuring Intracellular ATP using the same

Technical Field

The present invention relates to novel luciferase having resistance to a surfactant and a method for measuring intracellular ATP using the same.

Background Art

Intracellular ATP is routinely measured for determining the presence of cells in a sample or the number of cells in the fields of food sanitation, biology, clinical examinations, medical science, ultrapure water, and environmental science. A general method for measuring intracellular ATP comprises the steps of adding an ATP extraction reagent containing a surfactant as an effective component to a sample containing cells, extracting intracellular ATP, adding a luminescence reagent containing luciferase into the sample, and then measuring the total amount of light emitted.

Luciferase is an enzyme that catalyzes luminescence reaction of luciferin, which is a substrate, in the presence of ATP and magnesium ion. Luciferase used in a method for measuring intracellular ATP includes those derived from firefly species, such as GENJI firefly (*Luciola cruciata*), HEIKE firefly (*Luciola lateralis*), North American firefly and Russian firefly, etc.

Intracellular ATP can be extracted by adding an ATP extraction reagent to a sample containing cells and then stirring the sample.

To make full use of the capabilities of the extraction reagent, preferably the reaction agent is added so that the concentration of a surfactant becomes 0.05% or more of the mixture of the sample and the extraction reagent. However, a condition where the concentration of the surfactant is 0.05% or more, this inhibits significantly the enzyme

reaction in the process of measuring ATP concentration by bioluminescence. Thus the sensitivity and accuracy of measurement are largely impaired. This is because a surfactant at such a high concentration lowers luciferase activity.

For example, North American firefly luciferase activity decreases to about 20% in the presence of 0.1% benzalkonium chloride (See Table 1).

On the other hand, inhibition of the bioluminescent reaction can be reduced with a lower concentration of surfactant. However, in this case the extraction efficiency for ATP would be insufficient.

A method wherein cyclodextrin or its derivative is used is a known method for suppressing the inhibition of luminescence reaction by a surfactant (Japanese Patent Application Laid-Open No. 6-504200).

Among methods for measuring intracellular ATP wherein intracellular ATP is extracted by allowing a sample to contact with a surfactant and subsequently ATP is measured by luciferin-luciferase bioluminescent reaction method, a method for measuring intracellular ATP characterized by the application of the bioluminescent reaction method after allowing a sample, from which ATP is extracted, to contact with cyclodextrin (Japanese Patent Application Laid-Open Publication No. 7-203995) is also known.

There has been no attempt so far to suppress the inhibition of bioluminescent reaction due to a surfactant focusing on luciferase.

The purpose of the invention is to provide a novel luciferase having anti-surfactant resistance, whose activity is not impaired by the presence of a surfactant at a high concentration. The other purpose of the invention is to provide a method, comprising the steps of extracting intracellular ATP using a surfactant and measuring intracellular ATP by bioluminescent reaction using a luciferase, which can lower the inhibition of

bioluminescent reaction due to a surfactant without a decrease in efficiency in extracting intracellular ATP.

In the context of this Specification, the term "suppress" is used to describe significant reduction of the inhibition of the luminescence reaction by a surfactant and the complete elimination of this inhibition.

Disclosure of the Invention

The present invention relates to a luciferase having anti-surfactant resistance.

The luciferase having resistance to a surfactant includes a luciferase, wherein an amino acid at the 490-position, or an amino acid corresponding to the amino acid at 490-position of GENJI firefly or HEIKE firefly is substituted by an amino acid other than glutamic acid, e.g., lysine, in the amino acid sequence of a wild-type firefly luciferase.

Further, the luciferase having resistance to a surfactant includes a polypeptide consisting of (a) or (b):

- (a) A polypeptide consisting of the amino acid sequence shown in SEQ ID NO:4,
- (b) A polypeptide comprising additions, deletions, or substitutions of one or more of amino acids in the polypeptide of (a), and having luciferase activity resistant to a surfactant, or

a polypeptide consisting of (a) or (b):

- (a) A protein consisting of an amino acid sequence shown in SEQ ID NO:6,
- (b) A protein comprising additions, deletions, or substitutions of one or more of amino acids in the polypeptide of (a), and having luciferase activity resistant to a surfactant.

Further, the present invention relates to a luciferase gene encoding the luciferase having resistance to a surfactant.

Furthermore, the present invention relates to a recombinant vector containing the luciferase gene encoding the luciferase having resistance to a surfactant.

The present invention also relates to a transformant containing the recombinant vector.

In addition, the present invention relates to a method for producing the luciferase, comprising the steps of culturing the recombinant in a medium, and collecting luciferase with resistance to a surfactant from the culture product.

Moreover the present invention relates to a method for measuring intracellular ATP, comprising the steps of a first step wherein ATP is extracted in the presence of a surfactant from cells in a sample; a second step wherein a luminescence reagent containing luciferase is added to the extracted ATP solution so as to cause light emission; and a third step wherein the light emission is measured, and characterized in that luciferase having resistance to a surfactant is used.

This specification encompasses the description and/or drawings given in Japanese Patent Application No. H09-361022.

Brief Description of Drawings

Figure 1 shows a production processes for a mutant luciferase HIK.

Figure 2 shows change with time of light emission from natural type luciferase.

Figure 3 shows a comparative resistance against benzalkonium chloride of mutant luciferase.

Figure 4 shows a comparative resistance against benzetonium chloride of mutant luciferase.

Detailed Description of the Invention

The present invention will now be described in detail.

[Luciferase having resistance to surfactant]

Luciferase having resistance to a surfactant according to the present invention is as described below.

The term "having resistance to a surfactant" corresponds to any one of the following features.

- (1) When compared to known luciferase, the luciferase of the present invention leads to an increased initial amount of light emitted in the presence of a surfactant. Here the term "compare" means, for example, where the luciferase of the present invention is produced by introducing mutation into an amino acid sequence of known luciferase, to compare light emission from luciferase before and after the introduction of a mutation.
- (2) When compared to known luciferase, the luciferase of the present invention shows a gentle decrease in its activity in the presence of a surfactant.
- (3) The luciferase of the present invention has the remaining activity of more than 85% in the presence of 0.4% surfactant.

Hereinafter "luciferase having resistance to a surfactant" is referred to as "surfactant – resistant luciferase."

The term "activity" means the catalytic activity of bioluminescent reaction. Further any surfactant can be used in the present invention so far as it can be used in the measurement system for intracellular ATP. These surfactants include an anionic surfactant, cationic surfactant, ampholytic surfactant, non-ionic surfactant. A specific reagent is benzalkonium chloride or benzetonium chloride containing quaternary ammonium salt as a major component.

The luciferase of the present invention can be prepared from luminescence organs of luminescent organisms. The luminescent organisms include luminescent insects and

luminescent bacteria. The luminescent insects include those belonging to the order Coleoptera, such as those belonging to the family firefly and the family Pyrophorus. Specific examples include GENJI firefly, HEIKE firefly, North American firefly, Russian firefly, Pynophorus plagiophthalmus, Arachnocampa luminosa, and Rail worm. Further the luciferase of the present invention is obtained by cloning a luciferase gene from the luminescent organism and allowing the gene to express using an appropriate vector – host system.

Moreover, the luciferase of the present invention can be obtained by introducing mutation such as additions, deletions, and substitutions into an amino acid sequence of well-known luciferase. Well-known genetic engineering techniques can be used to introduce mutation into an amino acid sequence. In this case firstly, a mutation such as an addition, deletion, or substitution is introduced into a nucleotide sequence of a luciferase gene derived from the abovementioned luminescent organism or a well-known luciferase gene by genetic engineering techniques so as to generate a mutant luciferase gene. Subsequently, the mutant gene is incorporated into an appropriate host-vector system, thereby generating a recombinant microorganism. Then the recombinant microorganisms producing the luciferase of the present invention are selected by screening. The selected recombinant microorganisms are cultured in a medium. Finally the luciferase can be collected from the culture product.

Hereinafter surfactant-resistant luciferase obtained by introduction of a mutation into an amino acid sequence is referred to as “mutant luciferase.”

The mutant luciferase is for example, luciferase wherein an amino acid corresponding to an amino acid at the 490-position of the GENJI firefly luciferase or the HEIKE firefly luciferase, is substituted by an amino acid other than glutamic acid in an amino acid sequence of a wild-type firefly luciferase. The amino acid other than glutamic acid is a basic amino acid. Specific examples include lysine, arginine, and histidine. The

term "an amino acid corresponding to the amino acid at the 490-position of the GENJI or the HEIKE firefly luciferase" means an amino acid corresponding to the amino acid at the 490-position of the GENJI or HEIKE firefly luciferase when the determined amino acid sequence of luciferase is compared to an amino acid sequence of the GENJI or HEIKE firefly luciferase.

Moreover, in the GENJI or HEIKE firefly luciferase, the amino acid at the 490-position is glutamic acid. Further, in North American firefly luciferase, "an amino acid corresponding to the amino acid at the 490-position of the GENJI or the HEIKE firefly luciferase" corresponds to the glutamic acid at the 487-position.

More specifically, the mutant luciferase is a polypeptide comprising an amino acid sequence shown in SEQ ID NO:1 or 2, or said amino acid sequence wherein one or more amino acids are added, deleted or substituted.

[Method for producing mutant luciferase by genetic engineering techniques]

A method for generating mutant luciferase by genetic engineering techniques will now be described as follows.

The mutant luciferase is produced by introducing mutation such as additions, deletions, and substitutions into a nucleotide sequence of known luciferase and allowing an appropriate vector-host system to express the gene.

The known luciferase genes includes, but are not limited to, a firefly luciferase gene, more specifically a wild-type HEIKE firefly luciferase gene (Japanese Patent Application Laid-Open No. 2-171189) and a thermostable HEIKE firefly luciferase gene (Japanese Patent Application Laid-Open No. 5-244942).

i) A method for introducing mutation into a luciferase gene is, for example a

method wherein the gene and a mutagen are allowed to contact with each other. Specific examples of the mutagen include hydroxylamine, nitrous acid, sulfurous acid, and 5-bromouracil. Further, ultra violet irradiation, cassette mutagenesis, and site-directed mutagenesis using PCR can also be used. Furthermore, a mutant luciferase gene having a mutation at a desired position can be generated by annealing chemically synthesized DNA.

- ii) Next, the mutant luciferase gene is inserted into a vector DNA having such as a promoter sequence, a marker gene, and a replication origin, etc, thereby producing a recombinant plasmid. Any vector DNA can be used so far as it can be replicated in a host cell. Examples of the vector DNA include plasmid DNA and bacteriophage DNA. When the host cell is *Escherichia coli*, examples of the vector DNA include plasmid pUC119 (Takara Shuzo Co., Ltd.), pBluescript SK+(Stratagene), pMAL-C2 (NEW England Labs), pGEX-5X-1 (Pharmacia), pXa1 (Boehringer), and pMA56 (G.Ammerer, Meth. Enzymol., 101, 192, 1983).
- iii) Subsequently, an appropriate host cell is transformed or transduced with the above recombinant plasmid, and screening is performed for recombinant microorganisms having the ability to produce the mutant luciferase.

Any host cells including eucaryotic and prokaryotic cells can be used. The eucaryotic cells include animal, plant, insect, yeast cells. The prokaryotic cells include *Escherichia coli*, *Bacillus subtilis*, and *Actinomyces*. The animal cells include CHO, COS, HeLa cells and cells of myeloma cell lines. The prokaryotic cells include microorganisms belong to the genus *Escherichia*, such as *Escherichia coli* JM101 (ATCC 33876), JM109 (produced by Takara Shuzo Co., Ltd.), XL1-Blue (produced by Stratagene), and HB101 (ATCC33694).

Transformation in the present invention can be performed by for example, D.M. Morrison's method (Meth. Enzymol., 68, 326-331, 1979); Transduction can be

conducted by for example, B.Hohn's method (Meth. Enzymol., 68, 299-309, 1979).

Methods for purification of recombinant DNA from recombinant microorganisms include P.Guerry's method (J.Bacteriology, 116, 1064-1066, 1973), and D.B.Clewell's method (J.Bacteriology, 110, 667-676, 1972).

The nucleotide sequence of a gene inserted into the recombinant DNA can be determined by, for example Maxam-Gilbert method (Proc. Natl. Acad. Sci. USA, 74, 560-564, 1977), and Dideoxy method (Proc. Natl. Acad. Sci. USA, 74, 5463-5467, 1977).

- iv) The mutant luciferase of the present invention can be produced by culturing the recombinant microorganisms obtained in the manner described above in media.

When the host cell is *Escherichia coli*, recombinant *E.coli* may be cultured by solid culture methods, preferably liquid culture methods.

A culture medium of the present invention contains one or more nitrogen sources, such as yeast extract, tryptone, peptone, meat extract, corn steep liquor or exudate of soy bean or wheat bran, to which one or more of inorganic salts, such as sodium chloride, potassium phosphate, dipotassium phosphate, magnesium sulfate, magnesium chloride, ferric chloride, ferric sulfate or manganese sulfate are added. If necessary sugar and vitamins are added to this medium. Further the initial pH of the medium is preferably adjusted within pH 7 to 9. Moreover the culture is performed at a temperature within 30°C to 42°C, preferably at around 37°C for 3 to 24 hours, preferably for 5 to 8 hours. Preferable culture methods include aeration-agitation submerged culture, shaking culture, and static culture.

To recover mutant luciferase from the culture product after the completion of culturing recombinant *E.coli*, standard means for collecting enzymes can be employed. That is, the culture product is centrifuged to obtain cells. Then the cells are disrupted by treatment with lytic enzymes, such as lysozyme, ultrasonication, or milling. Fused

protein is discharged out of the cell. Subsequently insoluble substances are removed by filtration or centrifugation, so that a crude enzyme solution containing mutant luciferase can be obtained.

In the present invention the above crude enzyme solution can be used as authentic protein matter, or alternatively it can further be purified to higher purity by standard protein purification techniques. These techniques including sulfate salting out, organic solvent precipitation, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, adsorption chromatography, affinity chromatography, and electrophoresis can be used solely or in combination.

The use of surfactant-resistant luciferase of the present invention allows the addition of a surfactant at a high concentration in the extraction process for intracellular ATP.

[Detection of intracellular ATP of the present invention]

Detection of intracellular ATP of the present invention will be described as follows.

- i) First, ATP extraction reagent containing surfactant as an effective component is added to a sample containing cells so as to extract intracellular ATP out of the cells. The term "cells" refers to the cells derived from animal, plant, microorganism (e.g., yeasts, mold, fungi, bacteria, actinomyces, unicellular algae, viruses, and protozoa).

Any sample can be used so far as it contains the above cells. These samples include, but are not limited to, foods and drinks, pharmaceuticals, cosmetics, seawater, river water, industrial water, sewage, soil, urine, feces, blood, sputum, pus, and culture product of the above cells. A sample solution can also be prepared by suspending these samples in an appropriate solvent, such as distilled water, physiological saline, phosphoric acid buffer, Tris buffer, or sodium acetate

buffer. When a fluid specimen contains solids, the fluid specimen is suspended in an appropriate solvent or homogenized using a mixer so that it can be handled in the same manner as that in liquid form.

A sample of a filter membrane can also be prepared by filtering the above sample in liquid form through a hydrophilic or hydrophobic filter membrane.

The hydrophilic or hydrophobic filter membrane by which cells are captured can be used as a sample. In such a case, a film- or sheet-type hydrophilic filter membrane made of hydrophilic polytetrafluoroethylene, hydrophilic polyvinylidene fluoride, hydrophilic polyamide, acetylcellulose, and nitrocellulose, etc., can be used. Hydrophobic filter membranes made of PVDF (polyvinylidene fluoride), PTFE (polytetrafluoroethylene), and PE (polyethylene) etc., can be used.

Surfactants include anionic surfactants, cationic surfactants, ampholytic surfactants, and non-ionic surfactants.

Anionic surfactants include sodium dodecyl sulfate (SDS), lauryl potassium sulfate, sodium monolauryl phosphate, and sodium alkylbenzenesulfonic acid. Cationic surfactants include benzalkonium chloride (BAC), benzetonium chloride (BZC), cetylpyridinium chloride, cetyltrimethylammonium bromide, and myristyldimethylbenzylammonium chloride. ampholytic surfactants include Twittergent Detergent 3-08, 3-10, 3-12, 3-14, 3-16, and Tego. Finally non-ionic surfactants include Tween 20, 60, and 80, Span 60 and 80, Triton X-45 and x-100, polyoxyethylene ether, and polyoxyethylene lauryl ether.

Any concentration of a surfactant can be employed so far as it allows full expression of the ability to extract ATP. Preferable concentration of a surfactant is 0.05% or more of the mixture of a sample and ATP extraction reagent.

A sample and ATP extraction reagent are contacted with from each other at room temperature or with heating.

- ii) After ATP extraction, bioluminescent reagent is added to the sample containing surfactant-resistant luciferase so as to cause emission. Then the light emission is measured.

When surfactant-resistant luciferase is derived from a firefly, the bioluminescent reagents are those containing e.g., the following components (a) to (c).

- (a) surfactant-resistant luciferase
- (b) luciferin
- (c) magnesium ions or other metal ions

Further in addition to the above components, substances involving pH preparation or improved shelf life may be added. Such substances include EDTA 2Na, dithiothreitol, ammonium sulfate, sucrose, 2-mercaptoethanol, HEPES, Tricine, and Tris.

- iii) The amount of light emitted by the addition of a bioluminescent reagent can be measured by a luminometer such as a lumitester K-100 produced by Kikkoman Corporation, a luminescence reader BLR-201 produced by Aloka Co., Ltd. (an improved type, or a Lumat LB9501 produced by Berthold. When a filter membrane by which cells are captured is used as a sample, the cells can be counted using a bioluminescent image analysis system device to photograph spots on the filter membrane. Such a device is ARGUS-50/CL (with taper fiber: produced by Hamamatsu Photonics K.K.).

The present invention will now be described in detail by the use of examples.

However the technical field of the present invention is not limited by these examples.

Example 1 Surfactant resistance of natural type luciferase derived from various fire fly species.

(Method of preparing wild type luciferase derived from various firefly species)

Luciferase derived from GENJI and HEIKE fireflies was prepared according to the following methods. 1 mM ethylene diamine-4-acetate-2-sodium and 2mM phenylmethylsulfonylfluoride were added to 25mM Tris (hydroxy) aminomethane-hydrochloric acid buffer. Further ammonium sulfate was added to this solution so as to achieve 10% saturation. Tail portions of the various firefly species were added to this mixture at pH 7.8, and then disrupted using Hiskotoron (produced by Nichionrikakikaiseisakusho) . The resulting solution was centrifuged at 12,000 r.p.m. for 20 minutes to obtain supernatants as starting materials for purification. The purification was conducted by the process comprising salting out of ammonium sulfate, Ultrogel Ac A34 (produced by LKB) column, and hydroxy-apatite HPLC (produced byTOSHOH, TSK gel HA-1000) column. Finally an electrophoretically homogenous sample was obtained. In addition the luciferase derived from North American firefly is a commercial product (Sigma, L-9506).

(Method of determining luciferase activity)

A luciferase sample was properly diluted using enzyme-diluted solution (1mM EDTA, 1mM 2-mercaptoethanol, 1% BSA, 50mM HEPES, (pH7.5)). To 100 μ l of this solution, 100 μ l of substrate solution (1.4mM luciferin, 40mM ATP, 300mM MgSO₄. 7H₂O, 50mM HEPES, (pH 7.5)) was added.

The light emission was measured using BLE-201 Luminescence reader (produced by Aloka Co., Ltd.) under the following conditions.

Measuring range: x100

Numerical value displayed: x1000

Measuring temperature: 30°C

Measuring time: 20 seconds

1MLU (mega light unit) /ml is a value for activity when the measured value under these conditions was 1 Kcount.

(Method of determining surfactant-resistance)

Enzyme samples were obtained by preparing luciferase samples derived from various firefly species using enzyme-diluted solution (1mM EDTA, 1mM 2-mercaptoethanol, 5% glycerol, 50mM HEPES, (pH7.5)) to achieve 0.5 MLU/ml concentration.

50 μ l of 0.4% benzalkonium chloride (25mM Tricine at pH 7.75) and then 50 μ l of the enzyme sample were added to 100 μ l of substrate solution (4mM ATP, 0.4mM luciferin, 10mM magnesium sulfate, 50mM HEPES (pH 7.5)). After the solution was stirred for 5 seconds, the light emission was measured every second using Berthold Lumat LB-9501 for 1 minute.

Figure 2 shows the results. Along the vertical axis in this figure, the relative ratio of the light emission was plotted with the initial amount of light emitted considered to be 100% upon use of 25mM Tricine (pH 7.75) instead of 0.4% benzalkonium chloride.

As shown in these results, North American firefly luciferase was low in the initial light emission and the light emission decayed rapidly. This was caused by the low surfactant-resistance of the North American firefly luciferase. This can lead to low sensitivity and accuracy in measuring such values. On the other hand, GENJI firefly luciferase showed an initial light emission higher than that of North American firefly luciferase. That is, GENJI firefly luciferase was shown to have a surfactant resistance superior to that of North American firefly luciferase. Furthermore, HEIKE firefly luciferase showed an initial light emission higher than that of GENJI firefly luciferase

and the emission decayed slowly. Therefore, HEIKE firefly luciferase has good surfactant resistance, superior to that of GENJI firefly luciferase. These results suggest that the degree of surfactant resistance of luciferase varies according to the firefly species.

Example 2 Preparation of mutant luciferase HLK and HIK

Two types of mutant luciferase (named "HLK" and "HIK") were prepared according to the following methods.

(Production of a gene encoding mutant luciferase HLK)

A mutant luciferase gene was produced by site-directed mutagenesis using PCR. A plasmid pHLf7-217Leu described in Japanese Patent Application Laid-Open No. 5-244942 was used as a template for PCR reaction. The pHLf7-217Leu was a recombinant plasmid prepared by inserting a thermostable HEIKE firefly luciferase gene, in which an amino acid corresponding to Ala at the 217-position was substituted for a Leu-encoding gene, into a plasmid pUC119. In addition, E. coli JM101, to which the recombinant plasmid pHLf7-217Leu had been introduced, has been named E.coli JM101 (pHLf7-217Leu) and was deposited on April 22, 1992 as FERM BP-3840 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan).

The primer for PCR reaction was an oligonucleotide having a nucleotide sequence shown in SEQ ID No: 1 or 2. The DNA polymerase was a KOD dash polymerase (produced by TOYOBO). A PCR reaction cycle (94°C for 30 seconds, 50°C for 2 seconds, and 74°C for 3 minutes) was repeated for 30 times according to the examples attached to KOD dash polymerase. The PCR product was ligated into a circular recombinant plasmid pHLfLK using standard techniques.

Sequencing of a mutant luciferase gene contained in the pHLfLK was performed.

Reaction was conducted using a Diprimer Taq Sequencing Kit (produced by Applied Biosystems). Then the eletrophoretic analysis was performed using ABI 373A DNA sequencer (produced by Applied Biosystems). The entire nucleotide sequence of the obtained mutant luciferase gene is shown in SEQ ID NO: 3, and the amino acid sequence of a polypeptide encoded by this gene is shown in SEQ ID NO: 4. In the mutant luciferase gene, the genetic portion corresponding to alanine at the 217-position of wild-type HEIKE firefly luciferase was substituted by a gene encoding leucine, the genetic portion corresponding to glutamic acid at the 490-position of the same was substituted by a gene encoding lysine. The pHLfLK-introduced E.coli JM109 strain was named E.coli JM109 (pHLfLK) (see Figure 1). E. coli JM109 (pHLfLK) was deposited as FERM BP-6147 on October 16, 1997) with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

The polypeptide shown in SEQ ID NO:4 was named the mutant luciferase HLK.

(Preparation of gene encoding mutant luciferase HIK)

A mutant luciferase gene was prepared using the plasmid pHLf7-217Ile described in Japanese Patent Application Laid-Open No. 5-244942. The plasmid pHLf7-217Ile was a recombinant plasmid prepared by inserting a thermostable HEIKE firefly luciferase gene, in which an amino acid corresponding to Ala at the 217-position was substituted for a Ile-encoding gene, into a plasmid pUC119. The transformant strain obtained using this plasmid was deposited on April 22, 1992 as FERM BP-3841 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

About a 560bp fragment obtained by cutting the pHLfLK with EcoRV and NarI was obtained by agarose gel electrophoresis. Then the fragment was inserted into the pHLf7-217Ile treated with the same restriction enzymes.

The resulting recombinant plasmid has been named pHLfIK and the plasmid-introduced E.coli JM109 strain has been named E.coli JM109 (pHLfIK).

E.coli JM109 (pHLfIK) was deposited on October 16, 1997 as FERM BP-6146 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

The entire nucleotide sequence of the mutant luciferase gene contained in the pHLfIK is shown in SEQ ID NO: 5, and the amino acid sequence of a polypeptide encoded by this gene is shown in SEQ ID NO: 6. In the mutant luciferase gene, the genetic portion corresponding to alanine at the 217-position of wild-type HEIKE firefly luciferase was substituted by a gene encoding isoleucine, the genetic portion corresponding to glutamic acid at the 490-position of the same was substituted by a gene encoding lysine (see Fig. 1).

A polypeptide shown in SEQ ID NO:6 was named the mutant HIK firefly.

Example 3 Preparation of mutant luciferase HLK and HIK

E.coli JM109 (pHLfLK) and E.coli JM109 (pHLfIK) were inoculated on LB media (1% Bacto-trypton (W/V), 0.5% yeast extract (W/V), 0.5% NaCl (W/V), ampicillin (50 μ g/ml), 1.4% agar (W/V)), each containing ampicillin, and cultured at 37°C for 18 hours. The resulting culture fluid was centrifuged at 8000 r.p.m. for 10 minutes. The precipitated cells were suspended in 0.1M potassium phosphate buffer at pH 7.8 (0.1M ammonium sulfate, 1mM EDTA) were disrupted by ultrasonication.

Next, crude enzyme solution was obtained by centrifugation at 12000 r.p.m. for 10 minutes. The obtained enzyme solution was purified using the above purification techniques such that it becomes an electrophoretically homogenous sample.

Example 4 Surfactant resistance of mutant luciferase HLK and HIK

(Changes in emission with time)

To compare surfactant resistance of mutant luciferase with that of known luciferase, changes in emission with time were measured according to the aforementioned methods of measuring surfactant resistance. Figure 3 shows the results obtained by the use of 0.4% benzalkonium chloride (25mM Tricine (pH 7.75)). Figure 4 shows the results obtained by the use of 0.8% benzethonium chloride (25mM Tricine (pH 7.75)).

"HEIKE I mutant" in this figure is thermostable HEIKE firefly luciferase (described in Japanese Patent Application Laid-Open No. 5-244942) wherein Ala at the 217-position of wild-type HEIKE firefly luciferase is substituted for Ile. "HEIKE L mutant" is thermostable HEIKE firefly luciferase (Japanese Patent Application Laid-Open No. 5-244942) wherein Ala at the 217 position of wild-type HEIKE luciferase is substituted by Leu. "HIK" is a mutant wherein Glu at the 490-position of HEIKE I mutant is substituted by Lys, that is, the mutant luciferase HIK prepared in Example 3. "HLK" is a mutant wherein Glu at the 490-position of HEIKE L mutant is substituted by Lys, that is, the mutant luciferase HLK prepared in Example 3.

As can be seen in Fig. 3 which shows the results for benzalkonium chloride, the emission of HIK decayed more slowly than that of the HEIKE I mutant. Comparison of HLK and HEIKE L mutant reveals that HLK had initial light emission improved by about 20%, and slower decay in the light emission.

Therefore, the substitution of an amino acid at the 490-position resulted in improved surfactant-resistance of a luciferase.

As shown in Fig. 4 which shows the results obtained by the use of benzethonium chloride, HIK showed decay in emission more slowly than that of HEIKE I mutant. Further HLK showed slower decay in light emission than that of HEIKE L mutant. Therefore, the substitution of an amino acid at the 490-position resulted in improved

surfactant resistance.

(Comparison of emission rate)

The influence of the enzyme solution, substrate solution and benzalkonium chloride used when measuring change with time, on the measurement values taken under actual emission measurement conditions, was examined. Table 1 shows the light emission measured using Berthold Lumat LB-9501 under measuring conditions (5 seconds of waiting time, 3 seconds of measuring time).

In addition, the emission rate (remaining activity) was calculated by dividing the light emission measured in the presence of 0.4% benzalkonium chloride by a control value. Here the control value was the light emission upon use of 25mM Tricine at pH 7.75 instead of 0.4% benzalkonium chloride.

Table 1

Luciferase type	Light emission (RLU)		Emission rate (%)
	Without extraction reagent	With extraction reagent	
North American firefly	452563	97790	21.6
GENJI firefly	409406	167805	41.0
HEIKE firefly	425792	324724	76.3
HEIKE I mutant	422269	341039	80.8
HEIKE L mutant	423728	343634	81.1
HIK	386429	345159	89.3
HLK	390289	396764	101.7

North American firefly luciferase showed an emission rate as low as 21.6%, suggesting a large decrease in sensitivity. On the other hand, the emission rates for GENJI and HEIKE firefly luciferase were 41.0% and 76.3%, respectively, suggesting that the sensitivity of these firefly luciferases were less affected than that of North American firefly luciferase.

The emission rate for mutant luciferase HIK and HLK were 89.3% and 101.7%,

respectively. These rates were far greater than those of wild-type HEIKE firefly luciferase and thermostable HEIKE firefly luciferase. Particularly the emission rate of HLK was almost 100%. That is, HLK can yield the same light emission regardless of the presence or absence of a surfactant. Therefore, the sensitivity of HLK is totally unaffected by the use of a surfactant, allowing measurement with high accuracy.

(Comparison of IC₅₀)

Benzalkonium chloride and various luciferases were contacted with each other for 10 minutes. Then the benzalkonium chloride concentration (IC₅₀), at which activity is inactivated by 50% was determined. Equal amounts of luciferase solution prepared at this concentration and 0.01 to 0.1% benzalkonium chloride were mixed, and then allowed to stand for 10 minutes at room temperature. Subsequently, 100 μ l of substrate solution was added to the mixture. Immediately after addition, the light emission was measured using Berthold Lumat LB-9501. IC₅₀s obtained were as shown in Table 2.

Table 2 IC₅₀ for various luciferase

Luciferase type	IC ₅₀ (%)
North American firefly	0.014
GENJI firefly luciferase	0.016
HEIKE firefly luciferase	0.026
HEIKE I mutant	0.028
HEIKE L mutant	0.028
HIK	0.032
HLK	0.035

North American firefly luciferase showed the lowest IC₅₀ among the three types of wild-type luciferase. That is, North American firefly luciferase was shown to have the lowest resistance to a surfactant. HEIKE firefly luciferase showed the highest IC₅₀ among the wild-type luciferase. HLK and HIK showed IC₅₀ higher than those of wild-type HEIKE firefly luciferase and thermostable HEIKE firefly luciferase, suggesting that the resistance was improved by the substitution of an amino acid at the 490-position.

Especially HLK showed IC_{50} higher than that of HIK, indicating that HLK possesses the best surfactant-resistance.

Example 5 Method for measuring intracellular ATP

Next, a method for measuring intracellular ATP using the surfactant-resistant luciferase of the present invention will be described.

A standard technique used herein was TCA extraction method wherein intracellular ATP is extracted using trichloroacetic acid (TCA) and the amount of ATP extracted is measured using luciferin-luciferase luminescence reaction. TCA extraction method is excellent in extraction efficiency. Further in TCA extraction method no inhibition of luminescence reaction is caused by TCA because emission is measured after the sample containing TCA is diluted 1:100. Because of this dilution, however, TCA extraction method is complicated and can cause a decrease in the measuring sensitivity.

1. Materials

(1) Surfactant

Benzalkonium chloride (BAC, Japanese Pharmacopoeia) was used. ATP extraction reagent was prepared by dissolving this surfactant at 0.25% concentration into 25mM Tricine (pH 7.75).

(2) Microorganisms

Four strains, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29212) were used.

(3) Preparation of samples

In standard techniques, a sample, undiluted solution, was prepared by culturing the prescribed microorganisms on a normal broth medium (produced by Eiken chemical Co., Ltd.) at 35°C overnight. In the method of the present invention, a sample diluted solution was prepared by diluting an undiluted solution of the culture fluid to 1:100 with

sterile water.

(4) Luciferase

Surfactant-resistant luciferase of the present invention were HIK and HLK. Control surfactant-resistant luciferase types were known luciferase (North American firefly luciferase, GENJI firefly luciferase, HEIKE firefly luciferase, HEIKE I mutant, and HEIKE L mutant).

(5) Luminescence reagent

Luminescence reagent was prepared by adding various luciferase to solution containing 0.15mM luciferin, 6mM EDTA, 15mM magnesium acetate, 0.2mM dithiothreitol, 0.5% BSA and 25mM HEPES (pH 7.75).

The amount of luciferase to be added was prepared such that the light emission produced when 100 μ l of 2×10^{-8} M ATP standard solution was added to 100 μ l of the luminescence reagent would be the same amount of the light emission produced when a luminescence reagent attached to Luciferase LU (Kikkoman Corporation) was used.

2. Method for measuring intracellular ATP

(1) Method of the present invention

ATP extraction reagent 100 μ l was added to 100 μ l of a sample. The solution was allowed to stand for 20 seconds at room temperature. Then 100 μ l of the luminescence reagent was added to this solution. Immediately after addition, the light emission was measured using Lumat LB-9501 produced by Berthold.

(2) Standard technique

10 % trichloro acetate solution 100 μ l was added to 100 μ l of a sample and the solution was allowed to stand for 1 minute. 25 mM Tricine (pH 7.75) 9.8ml was added to this extract, and then the extract was well stirred. 25 mM Tricine (pH 7.75) and 100 μ l of a luminescence reagent attached to CheckLite LU (produced by Kikkoman Corporation) were added to 100 μ l of the sample. Immediately after addition, the light emission was measured using Lumat LB-9501 produced by Berthold.

3. Results

Tables 3 and 4 show the results. The relative ratio of the light emissions obtained by the use of the luminescence reagents using various luciferase types is also shown in these tables. Here the light emission obtained by the standard technique (TCA extraction method) was defined as 100%.

Table 3 Detection of intracellular ATP

Measuring method	E.coli ATCC25922		S.aureus ATCC 25923	
	Measured value (RLU)	Relative ratio (%)	Measured value(RLU)	Relative ratio (%)
Standard technique (TCA extraction method)	132794	(100.0)	130220	(100.0)
North American firefly	153	(0.1)	163	(0.1)
GENJI firefly luciferase	463	(0.3)	659	(0.5)
HEIKE firefly luciferase	76082	(57.3)	74019	(56.8)
HEIKE I mutant	47655	(35.9)	50031	(38.4)
HEIKE L mutant	46217	(34.8)	51243	(39.4)
HIK	97073	(73.1)	76533	(58.8)
HLK	87981	(66.3)	72182	(55.4)

Table 4 Detection of intracellular ATP

Measuring method	<i>P.aeruginosa</i> ATCC 27853		<i>E.faecalis</i> ATCC 29212	
	Measured value (RLU)	Relative ratio (%)	Measured value(RLU)	Relative ratio (%)
Standard technique (TCA extraction method)	168141	(100.0)	12427	(100.0)
North American firefly	553	(0.3)	113	(0.1)
GENJI firefly luciferase	1503	(0.9)	163	(1.3)
HEIKE firefly luciferase	117096	(69.6)	8132	(65.4)
HEIKE I mutant	80455	(47.8)	4586	(36.9)
HEIKE L mutant	81069	(48.2)	4762	(38.3)
HIK	131134	(78.0)	7914	(63.7)
HLK	131815	(78.4)	7998	(64.4)

No emission was observed for the luminescence reagent containing North American firefly luciferase. GENJI firefly luciferase showed weak emission. This is because the luciferase itself was devitalized by the surfactant. Therefore, it was shown that the surfactant at high concentration such as was used in this examination cannot be used as an ATP extraction reagent for the luciferase.

Unlike North American firefly luciferase and GENJI firefly luciferase, HEIKE firefly luciferase showed emission 60 to 70% of that in TCA extraction method. HEIKE firefly luciferase was shown to possess surfactant-resistance higher than those of North American firefly luciferase and GENJI firefly luciferase.

Light emissions from HEIKE L mutant, and HEIKE I mutant which is thermostable HEIKE firefly luciferase were each equivalent to around 40% of that in TCA extraction method, and largely lower than that of wild-type HEIKE firefly luciferase.

Each of the light emission from HIK and HLK, which is surfactant-resistant luciferase

of the present invention, respectively was more intense than that from wild-type HEIKE luciferase and thermostable luciferase. Further the light emission in this case was equivalent to 60 to 80% of that in TCA extraction method.

HIK and HLK are mutants wherein Glu at the 490-position of HEIKE I and HEIKE L mutants are substituted for Lys, respectively. That is, the introduction of said mutation into the amino acid at the 490-position improved resistance to a surfactant. The sensitivity of HIK and HLK is less affected by ATP extraction reagent even at such a high concentration employed in this examination, suggesting the use of HIK and HLK enable highly accurate measurement.

Industrial Applicability

The use of a novel surfactant-resistant luciferase according to the present invention for measuring intracellular ATP allows the detection without a decrease in luciferase activity even in the presence of a surfactant at a high concentration.

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

Sequence Listing Free Text

SEQ ID NO:1: A synthetic DNA

SEQ ID NO:2: A synthetic DNA